EXHIBIT 11

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Antiplatelet and Antithrombotic Effects of a Novel Selective Phosphodiesterase 3 Inhibitor, NSP-513, in Mice and Rats

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ABSTRACT—We investigated the effects of NSP-513, (*R*)-4,5-dihydro-5-methyl-6-[4-(2-propyl-3-oxo-1-cyclohexenyl)amino] phenyl-3(2*H*)-pyridazinone, on phosphodiesterase (PDE) isozyme activities, in vitro platelet aggregation and in vivo thrombus formation. NSP-513 selectively inhibited human platelet PDE 3 isozyme with an IC_{50} value of 0.039 μM. In an in vitro human platelet aggregation assay, the IC_{50} values (μM) of NSP-513 for platelet aggregation induced by collagen, U-46619, arachidonic acid, adenosine diphosphate (ADP), epinephrine and thrombin were 0.31, 0.25, 0.082, 0.66, 0.23 and 0.73, respectively. In a mouse pulmonary thromboembolism model, orally administered NSP-513 showed in vivo antithrombotic effects that were 320 to 470 times more potent than those of cilostazol. In a rat carotid arterial thrombosis model, intraduodenally administered NSP-513 (0.1 mg/kg), cilostazol (30 mg/kg) and aspirin (30 mg/kg) reduced thrombus formation by 75%, 66% and 48%, respectively. However, intravenously administered dipyridamole (10 mg/kg) did not significantly prevent thrombus formation. These results demonstrate that NSP-513 has the potential to prevent not only in vitro platelet aggregation but also in vivo thrombus formation and indicate that the highly selective PDE 3 inhibitory effect of NSP-513 may make this compound useful for assessing the physiological role of PDE 3.

Keywords: NSP-513, Phosphodiesterase 3, Arterial thrombosis, Platelet aggregation

Several platelet activators are released from injured arterial walls (1 – 3). Activated platelets then adhere to the disrupted vessel surface, and the resulting aggregation is associated with thrombus formation. It is widely accepted that thrombus formation affects the progression of symptoms in various cardiovascular or cerebrovascular disorders such as unstable angina, myocardial infarction, transient ischemic attack and atherosclerosis (4 – 8). Aspirin is the most commonly used antiplatelet drug. However, it is believed that several platelet activators, such as adenosine diphosphate (ADP), thrombin, epinephrine and thromboxane A_2 (TXA_2), simultaneously contribute to thrombus formation. For this reason, new antiplatelet drugs have been developed for the treatment of thrombus formation in patients with vascular disorders.

Multiple forms of cyclic nucleotide phosphodiesterase (PDE) have been isolated from many tissues and have been classified largely into seven isozyme families (PDE 1 through 7) (9, 10). The results of in vitro studies using platelets suggested that selective PDE 3 inhibition increased intracellular cyclic adenosine monophosphate (cAMP) levels, decreased calcium levels and prevented platelet aggregation by several platelet activators such as collagen, arachidonic acid, ADP and epinephrine (11 – 14). However, there are few reports regarding the in vivo antithrombotic activities of selective PDE 3 inhibitors, especially in an arterial thrombosis model with actual thrombi on the inner surface of blood vessels. There are also few comparative studies of selective PDE 3 inhibitors versus other antiplatelet drugs such as aspirin. PDE isozymes are present not only in platelets but also in blood vessels, and in vivo thrombus formation is affected by cardiovascular conditions including blood flow or endothelial status. Therefore, it is important to evaluate the in vitro antiplatelet and in vivo antithrombotic activity of selective PDE 3 inhibitors.

We performed conformational analysis on cAMP analogues and PDE isozymes with computer modeling and obtained NSP-513, (*R*)-4,5-dihydro-5-methyl-6-[4-(2-propyl-3-oxo-1-cyclohexenyl)amino] phenyl-3(2*H*)-pyridazinone,

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as a promising inhibitor (15). To determine whether a selective PDE 3 inhibitor such as NSP-513 prevents in vivo thrombosis induced by various platelet aggregating agents as well as in vitro platelet aggregation, we compared the in vivo antithrombotic effects of NSP-513 and cilostazol (14, 16), selective PDE 3 inhibitors, with those of aspirin, a cyclooxygenase (COX) inhibitor, in a murine model of pulmonary thromboembolism induced by arachidonic acid, ADP or collagen plus epinephrine. Electrical stimulation-induced injury of blood vessels has long been used to induce experimental thrombus (17–21); such a thrombus induced in an artery is morphologically consistent with human thrombi in disorders involving arterial thrombosis (18, 21). Therefore, to determine whether the inhibition of PDE 3 alone can inhibit thrombus formation in injured blood vessels, we also compared the in vivo antithrombotic activity of NSP-513 with those of cilostazol, dipyridamole and aspirin in a rat model of electrical stimulation-induced carotid arterial thrombosis.

MATERIALS AND METHODS

All experiments complied with the Guidelines for Biological and Pharmacological Experiments approved by Tsukuba Research Institute of Banyu Pharmaceutical Co., Ltd. and the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. For human blood sampling, healthy volunteers were informed of the purpose of this study and were paid for their participation.

Drugs

NSP-513 was synthesized at Nippon Soda Co., Ltd. (Odawara). Cilostazol was extracted and purified from Pletaal® tablets (Otsuka Pharmaceutical Co., Tokyo). Aspirin, ADP and heparin were purchased from Wako Pure Chemical Industries Ltd. (Osaka). Milrinone, U-46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$), arachidonic acid and thrombin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Collagen was purchased from Nycomed (Munich, Germany). Epinephrine (Bosmin®) was purchased from Daiichi Pharmaceutical Co. (Tokyo). All agents were prepared just before use.

In vitro PDE activity assay

PDE activity was measured by the method of Thompson et al. (22) with minor modifications.

Beagle dogs were anesthetized, and each animal's heart and kidneys were rapidly excised and placed in ice-cold saline. The left ventricle and kidney cortex were minced, frozen rapidly in liquid nitrogen and stored at −80°C. Human blood from healthy volunteers was collected by venipuncture into Terumo Single Blood Bags (Terumo, Tokyo) containing trisodium citrate (0.37% final concentration). Platelet-rich plasma (PRP) was obtained from whole blood by centrifugation at $110 \times g$ for 10 min at room temperature. A platelet pellet was obtained by further centrifugation of the PRP at $1,050 \times g$ for 10 min at 4°C. The pellet was resuspended in a volume of Tris-buffered saline (20 mM Tris-HCl, 135 mM NaCl, 5 mM glucose, 13 mM sodium citrate, 2 mM EDTA-2Na, pH 7.4 at 4°C) equal to the original PRP volume and precipitated by centrifugation at $1,050 \times g$ for 10 min at 4°C. This washing procedure was performed 3 times. The pellet was stored at −80°C until use.

The different molecular forms of PDE from the canine left ventricle and kidney cortex and from human platelets were separated by DEAE-cellulose anion exchange liquid chromatography. Briefly, the tissues and platelets stored at −80°C were thawed, minced and homogenized in 10 vol of buffer A (10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 μM leupeptin, 1 μM pepstatin A, 1 μM *p*-amidinophenylmethanesulphonyl fluoride (p-APMSF) and 1 mM dithiothreitol) with a polytron homogenizer. This and all subsequent procedures were performed under ice-cold conditions. The homogenate was sonicated and then centrifuged at $100,000 \times g$ for 40 min. The supernatant was filtered through 4 layers of gauze and dialyzed for 5 h against 5 l of buffer B (70 mM sodium acetate, pH 6.5, 5 mM 2-mercaptoethanol, 1 μM leupeptin, 1 μM pepstatin A and 1 μM p-APMSF). The dialyzed supernatant was then applied to a DEAE-cellulose column (DE-52; Whatman, Kent, UK) equilibrated with buffer B. After the column was washed with buffer B, PDE was eluted from the column using a continuous 70–1,000 mM sodium acetate gradient buffer solution (containing 5 mM 2-mercaptoethanol, 1 μM leupeptin, 1 μM pepstatin A, 1 μM p-APMSF, pH 6.5; total volume of 400 ml and flow rate of 20 ml/h). A 5-ml aliquot of each of the eluted solutions was collected. Appropriate peak fractions (referred to as PDE 1, 2, 3, 4 and 5) were pooled, concentrated using an Amicon ultrafiltration cell fitted with a YM-10 membrane (Millipore Corp., Bedford, MA, USA), and dialyzed against buffer B for 24 h. The enzyme solution was diluted to 50% with glycerol and stored at −20°C.

The fractions were assayed for cAMP- and cGMP-PDE activity (substrate concentration: 1 μM) in the presence or absence of 0.3 units of calmodulin and 10 μM $CaCl_2$ or 1 μM cGMP. The identity of the isozymes was confirmed by their sensitivity to Ca^{2+}/calmodulin (activator of PDE 1) and cGMP (activator of PDE 2, inhibitor of PDE 3 but not PDE 4, and specific substrate for PDE 5). The reaction medium consisted of 40 mM Tris-HCl, 2 mM $MgSO_4$ and 0.5 mM 2-mercaptoethanol (pH 7.5). The concentration of substrate ([^{3}H]-cAMP or [^{3}H]-cGMP) was 1 μM. By suit-

able enzyme dilution, the rate of hydrolysis was kept to less than 20% of the added substrate. The reaction mixture was incubated at 30°C for 30 min and stopped by exposure to a boiling water bath for 1 min. After cooling and the addition of 100 μl of snake venom solution (1 mg/ml), the mixture was incubated for 15 min at 30°C. The formed [^{3}H]-adenosine or [^{3}H]-guanosine was separated from the reaction mixture by a small anion exchange resin column (AG1-X8; Bio-Rad, Hercules, CA, USA). Radioactivity was determined by a liquid scintillation counter. Each assay was performed in triplicate for the same reaction mixture. The concentration causing 50% inhibition (IC_{50}) of PDE activity was calculated from the concentration-inhibition curve. The inhibitory effect on cardiac PDE 3 activity was measured in the presence of 30 μM rolipram, a PDE 4 inhibitor, to prevent the influence of contamination with PDE 4. The drugs were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in the medium was 2%, which inhibited PDE activity by less than 10%.

In vitro platelet aggregation assay

Blood was collected from human volunteers by venipuncture and from rats by aortic puncture, and it was drawn into a plastic syringe containing 3.8% (human) and 3.0% (rat) trisodium citrate solution (1:9 citrate/blood, v/v). The healthy, male volunteers had not taken any drugs (including aspirin) for at least 7 days. Male SD rats were purchased from Charles River Japan, Inc. (Kanagawa). PRP was prepared by centrifugation at room temperature for 15 min at 110 × *g* for human blood and 135 × *g* for rat blood. Platelet-poor plasma (PPP) was obtained from the precipitated fraction of PRP by centrifugation at room temperature for 15 min at 1,050 × *g*. The platelet count in PRP was adjusted to 3×10^8 platelets/ml using PPP.

Platelet aggregation was induced by adding a submaximal concentration of the respective agonists and continuously measured by recording the light transmission with a PAM-8C aggregometer (Mebanix, Tokyo). A 200-μl sample of PRP was placed in the cuvette of the aggregometer and preincubated for 0.5 min at 37°C. In the case of thrombin, a washed platelet suspension was used and 2 μl of 100 mM $CaCl_2$ was added to the suspension just before preincubation. Then, 1 μl of NSP-513, cilostazol or aspirin dissolved and diluted in DMSO or vehicle solution was added to the PRP. After 3 min of preincubation at 37°C, 10 μl of each aggregating agent was added to the reaction medium. The light transmission on the aggregation response curve was measured for 7 min. The following aggregating agents were used in 0.9% NaCl saline solutions prepared immediately before use: U-46619, arachidonic acid, ADP, epinephrine and thrombin; collagen was diluted in Suspension Kollagen Fibrils (SKF) buffer (Nycomed). The maximum aggregation on the aggregation response curve was taken as a measure of the extent of platelet aggregation, and the concentration causing 50% inhibition (IC_{50}) of platelet aggregation was calculated from the concentration-inhibition curve.

In vivo pulmonary thromboembolism model

Sudden death associated with platelet aggregation in the pulmonary microcirculation was induced in mice by treatment with platelet-aggregating agents (23–25). Male ICR mice weighing 20–30 g (Charles River Japan, Inc.) were used in this experiment. Mice were fasted overnight before the tests. Acute pulmonary thromboembolism was induced by i.v. injection of the following platelet-aggregating agents: arachidonic acid (90 mg/kg), ADP (400 mg/kg), and collagen (500 μg/kg) plus epinephrine (50 μg/kg). These agents were dissolved or diluted in saline to yield an administration volume of 100 μl/10 g body weight. NSP-513 (0.03, 0.1, 0.3, 1, 3 and 10 mg/kg), cilostazol (30, 100, 300 and 900 mg/kg) or aspirin (10, 30, 100, 300 and 900 mg/kg) was administered orally to the animals 1 h before i.v. injection of the platelet aggregating agents. NSP-513, cilostazol and aspirin were suspended in 0.5% methyl cellulose (MC) to yield an administration volume of 100 μl/10 g body weight. The control mice received vehicle (0.5% MC) alone. The mortality rate was determined for 30 min after injection of the aggregating agents.

In vivo electrical stimulation-induced carotid arterial thrombosis model

Thrombi were produced in male SD rats weighing 400–450 g (Charles River Japan, Inc.) by an electrical stimulation method modified from that of Schumacher et al. (21), as follows: After overnight fasting, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p. injection followed by a 20 mg/kg per hour i.v. infusion). Polyethylene cannulas were inserted into the jugular vein or the duodenum to administer the drug, into the femoral artery to monitor mean blood pressure and heart rate, and into the trachea to maintain airway patency. The right carotid artery was exposed and dissected free of nerves and connective tissue. A piece of Parafilm® was placed under the entire length of the exposed vessel to provide electrical isolation. An electromagnetic flow probe (1.0-mm diameter; Nihon Kohden, Tokyo) was placed on the right carotid artery, and carotid blood flow was monitored continuously (MFV-3100, Nihon Kohden). A stainless steel L-shaped needle (30 G, 5-mm tip) was inserted into the carotid artery and placed in contact with the intimal surface of the vessel downstream from the flow probe about 60 min before the application of current. An anodic current of 1 mA was delivered for 6 min using a constant current stimulator (SEN-3201, Nihon Kohden) and an isolator (SS-102J, Nihon Kohden). The cathode was a silver hook-

shaped wire attached to the carotid artery. At 40 min after the start of the anodic current, the artery was opened lengthwise and the thrombus was removed. Wet weight was determined immediately using an electronic balance (H110; Sartorius Co., Göttingen, Germany). Changes in blood flow were expressed as a percentage of the basal blood flow before drug administration.

NSP-513 (0.01, 0.03 and 0.1 mg/kg), cilostazol (0.3, 3, 30 mg/kg) or aspirin (10, 30 and 100 mg/kg) was injected intraduodenally (i.d.) 30 min before the application of current. For i.d. injection, aspirin, NSP-513 and cilostazol were suspended in 0.5% MC to yield an administration volume of 5 ml/kg. Dipyridamole (3 and 10 mg/kg) was injected i.v. 15 min before the application of current. For i.v. injection, dipyridamole was dissolved in saline solution to yield an administration volume of 1 ml/kg. Intravenous infusion of heparin (300 units/kg per hour) was started 15 min before the application of current. Heparin was dissolved or diluted in saline to provide an infusion rate of 1 ml/h. The control rats received vehicle alone.

Statistical analyses

Data are expressed as the mean alone or the mean ± S.E.M. Statistical analyses were performed by Fisher's exact test for the pulmonary thromboembolism model and Dunnett's test after analysis of variance for the carotid thrombosis model. P values less than 0.05 were considered significant.

RESULTS

Inhibition of PDE isozyme activity

The inhibitory effects of NSP-513, cilostazol and dipyridamole were investigated for PDE 2, PDE 3 and PDE 5 in human platelets; for PDE 1, PDE 2 and PDE 3 in canine left ventricle; and for PDE 4 in canine kidney cortex. In order of potency, the IC_{50} values of the tested drugs for the inhibition of human platelet PDE 3 were 0.039 μM for NSP-513, 0.50 μM for cilostazol and 100 μM for dipyridamole (Table 1). The inhibitory effect of NSP-513 on PDE 3 was 13 and 2,600 times more potent than those of cilostazol and dipyridamole, respectively. In addition, NSP-513 was at least 850 times more potent against human platelet PDE 3 than against other PDE isozymes (1, 2, 4 and 5). The selectivity ratio of cilostazol for human platelet PDE 3 as compared with the other isozymes ranged from 11 to >200. In contrast, dipyridamole was at least 100 times more selective against human platelet PDE 2 and 5 than against human platelet PDE 3.

Effects on in vitro platelet aggregation induced by various platelet activators

NSP-513 showed potent and concentration-dependent inhibition of human platelet aggregation induced by collagen (3 μg/ml), U-46619 (3 μM), arachidonic acid (300 μg/ml), ADP (10 μM), epinephrine (10 μM) and thrombin (0.3 units/ml), with IC_{50} values (μM) of 0.31, 0.25, 0.082, 0.66, 0.23 and 0.73, respectively (Table 2). In addition, the IC_{50} value (μM) of NSP-513 for rat platelet aggregation induced by collagen (30 μg/ml) was 0.49. The antiplatelet effect of NSP-513 was 11 to 65 times more potent than that of cilostazol. NSP-513 and cilostazol completely inhibited both the first and second phases of platelet aggregation induced by ADP and by epinephrine. However, aspirin inhibited only the second phase of ADP- and epinephrine-induced aggregation. Moreover aspirin did not produce a 50% inhibition of the aggregation induced by U-46619, ADP or thrombin even at the highest concentration of 1 mM.

Table 1. Inhibition of PDE isozymes by NSP-513, cilostazol and dipyridamole

Drugs	IC_{50} (μM) and selectivity against human platelet PDE 3						
	Human platelets			Dog left ventricle			Dog kidney cortex
	PDE 2	PDE 3	PDE 5	PDE 1	PDE 2	PDE 3	PDE 4
NSP-513	45 (1200)	0.039 (1)	33 (850)	>1000 (>26000)	500 (13000)	0.064 (1.6)	41 (1100)
Cilostazol	5.6 (11)	0.50 (1)	23 (46)	>100 (>200)	58 (120)	1.2 (2.4)	78 (160)
Dipyridamole	0.75 (0.008)	100 (1)	0.97 (0.01)	76 (0.76)	30 (0.3)	240 (2.4)	3.9 (0.04)

50% inhibitory concentration (IC_{50}) values were determined by least-squares linear regression analysis from the percent inhibition of phosphodiesterase (PDE) activity versus the log molar concentrations of drugs. The values in parentheses indicate the selectivity of inhibition against human platelet PDE 3. Each assay was performed in triplicate for the same reaction mixture.

Table 2. Inhibitory effects of NSP-513, cilostazol and aspirin on human platelet aggregation induced by various aggregating agents

Drugs	IC_{50} (μM)					
	Collagen	U-46619	AA	ADP	Epi	Thrombin
NSP-513	0.31 ± 0.02	0.25 ± 0.08	0.082 ± 0.029	0.66 ± 0.11	0.23 ± 0.05	0.73 ± 0.30
Cilostazol	20 ± 2	15 ± 3	4.4 ± 0.9	31 ± 5	6.4 ± 1.3	8.0 ± 4.5
Aspirin	109 ± 7	>1000	17 ± 0.2	>1000	45 ± 15	>1000

Platelets were incubated with various concentrations of each drug for 3 min before the addition of 3 μg/ml of collagen, 3 μM of U-46619, 300 μg/ml of arachidonic acid (AA), 10 μM of adenosine diphosphate (ADP), 10 μM of optnephrine (Epi) or 0.3 units/ml of thrombin. 50% inhibitory concentration (IC_{50}) values were determined by least-squares linear regression analysis from the percent inhibition of platelet aggregation versus the log molar concentrations of drugs. Data are presented as the mean ± S.E.M. of 3–6 preparations.

Effects on in vivo pulmonary thromboembolism induced by various platelet activators

Orally administered NSP-513 dose-dependently inhibited the sudden death accompanied by acute pulmonary thromboembolism that was induced by arachidonic acid, ADP or collagen plus epinephrine; and the inhibitory effects of NSP-513 were 320 to 470 times more potent than those of cilostazol. In the model of arachidonic acid-induced sudden death, the ID_{50} values of NSP-513, cilostazol and aspirin were 0.12, 56 and 41 mg/kg, respectively (Table 3). In the model of ADP-induced thromboembolism, the ID_{50} values of NSP-513 and cilostazol were 0.45 mg/kg and 160 mg/kg, respectively (Table 4). In contrast, aspirin had no effect even at 300 mg/kg. In the model of collagen-plus-epinephrine-induced thromboembolism, the ID_{50} value of NSP-513 was 2.8 mg/kg, and cilostazol at 900 mg/kg produced about 50% inhibition (Table 5). However, aspirin even at 900 mg/kg produced only 10% inhibition.

Effects on in vivo electrical stimulation-induced carotid arterial thrombosis

We induced occlusive thrombosis with the carotid blood flow (CBF) decreased to zero within at least 30 min after the application of anodic current. Wet thrombus weight did not differ significantly among the vehicle-treated control groups and averaged 4.8 ± 0.2 mg (N = 15) overall. Sham operation without applied current demonstrated that CBF was maintained throughout the experiment, but minor thrombi were observed around the inserted electrode. Thrombus weight in the vehicle-treated sham-operation groups averaged 0.6 ± 0.1 mg (N = 15) overall. Therefore, in the following study, the % inhibition of thrombus

Table 3. Effects of NSP-513, cilostazol and aspirin on arachidonic acid-induced pulmonary thromboembolism in mice

Drugs	Dose (mg/kg)	Dead/Tested	% Inhibition	ID_{50} (mg/kg)
Vehicle	—	26/30	—	—
NSP-513	0.03	16/20	8	
	0.1	11/20[b]	37	
	0.3	3/20[a]	83	0.12
	1	4/20[a]	77	
	3	0/20[a]	100	
Cilostazol	10	14/20	19	
	30	10/20[a]	42	56
	100	8/20[a]	54	
	300	3/20[a]	83	
Aspirin	10	16/20	8	
	30	9/20[a]	48	
	100	5/20[a]	71	41
	300	6/20[a]	65	

Drugs were administered orally 1 h before i.v. injection of arachidonic acid (90 mg/kg). 50% inhibitory dose (ID_{50}) values were determined by least-squares linear regression analysis from the percent inhibition of mortality versus the log molar doses of drugs. Dead = number of dead mice, Tested = number of tested mice. [a]P<0.01, [b]P<0.05 versus vehicle.

Table 4. Effects of NSP-513, cilostazol and aspirin on ADP-induced pulmonary thromboembolism in mice

Drugs	Dose (mg/kg)	Dead/Tested	% Inhibition	ID_{50} (mg/kg)
Vehicle	—	20/20	—	—
NSP-513	0.3	11/20[a]	45	
	1	8/20[a]	60	0.45
	3	6/20[a]	70	
Cilostazol	30	14/20[b]	30	
	100	12/20[a]	40	160
	300	8/20[a]	60	
Aspirin	30	20/20	0	
	100	20/20	0	>300
	300	20/20	0	

Drugs were administered orally 1 h before i.v. injection of ADP (400 mg/kg). 50% inhibitory dose (ID_{50}) values were determined by least-squares linear regression analysis from the percent inhibition of mortality versus the log molar doses of drugs. ADP = adenosine diphosphate, Dead = number of dead mice, Tested = number of tested mice. [a]P<0.01, [b]P<0.05 versus vehicle.

Table 5. Effects of NSP-513, cilostazol and aspirin on collagen-plus-epinephrine-induced pulmonary thromboembolism in mice

Drugs	Dose (mg/kg)	Dead/Tested	% inhibition	ID_{50} (mg/kg)
Vehicle	—	30/30	—	—
NSP-513	0.3	18/20	10	
	1	13/20*	35	
	3	12/20*	40	2.8
	10	4/20*	80	
Cilostazol	30	19/20	5	
	100	18/20	10	>900
	300	15/20*	25	
	900	11/20*	45	
Aspirin	30	18/20	10	
	100	15/20*	25	
	300	16/20[b]	20	>900
	900	18/20	10	

Drugs were administered orally 1 h before i.v. injection of collagen (500 μg/kg) plus epinephrine (50 μg/kg). 50% inhibitory dose (ID_{50}) values were determined by least-squares linear regression analysis from the percent inhibition of mortality versus the log molar doses of drugs. Dead = number of dead mice, Tested = number of tested mice. *P<0.01, [b]P<0.05 versus vehicle.

formation for each drug was calculated by discounting the thrombus weight observed in the sham-operation groups.

Intraduodenal injection of NSP-513 (0.01 – 0.1 mg/kg) or cilostazol (0.3 – 30 mg/kg) prevented CBF reduction and thrombus formation in a dose-dependent manner (Figs. 1 and 2). In the NSP-513 (0.1 mg/kg)-treated and cilostazol (30 mg/kg)-treated groups, thrombus weights were 1.6 ± 0.2 mg (74.5% inhibition, P<0.01) and 1.9 ± 0.3 mg (66% inhibition, P<0.01), respectively. However, i.v. injection with dipyridamole at doses of 3 and 10 mg/kg did not significantly prevent CBF reduction or thrombus formation (Fig. 3).

NSP-513 did not affect mean blood pressure or heart rate, but the highest dose of NSP-513 (0.1 mg/kg) showed a tendency to slightly, although not significantly, increase heart rate (Fig. 4). Similarly, cilostazol and dipyridamole did not significantly affect mean blood pressure or heart rate (data not shown).

Intraduodenal injection of aspirin significantly prevented CBF reduction and thrombus formation, although dose-dependency was not observed (Fig. 5). The maximal antithrombotic effect of i.d. aspirin was observed at an intermediate dose of 30 mg/kg at a thrombus weight of 2.7 ± 0.4 mg (48.1% inhibition, P< 0.05).

Heparin (100 and 300 units/kg per hour, i.v. infusion) prevented CBF reduction and thrombus formation in a dose-dependent manner (Fig. 6). Thrombus weight decreased by 52.7% with heparin at a dose of 300 units/kg per hour (P<0.05).

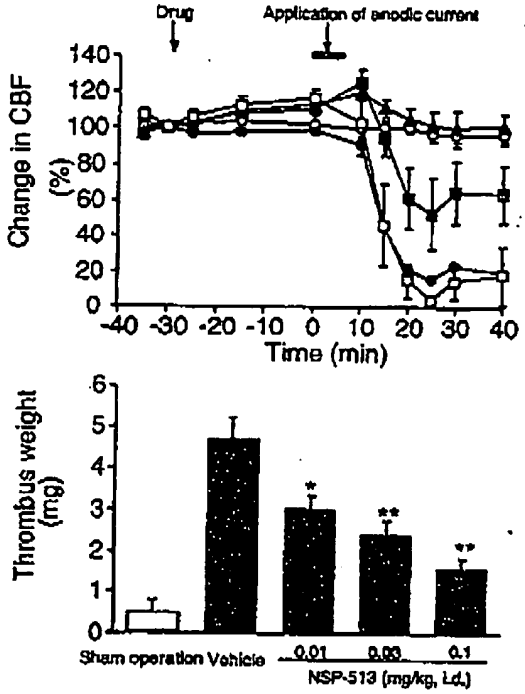

Fig. 1. Effects of intraduodenally (i.d.) injected NSP-513 on carotid arterial blood flow (CBF) (top) and thrombus weight (bottom) in an electrical stimulation-induced carotid arterial thrombosis rat model. NSP-513 (0.01 – 0.1 mg/kg, i.d.) or vehicle was injected 30 min before the application of anodic current (1 mA, 6 min). Changes in CBF are expressed as a percentage of the basal CBF measured just before injection in each rat. The basal CBF values were 7.2 ± 1.1 ml/min (sham ope., ○), 6.5 ± 0.8 ml/min (vehicle, □), 8.9 ± 1.0 ml/min (NSP-513 at 0.01 mg/kg, ●), 8.7 ± 1.2 ml/min (NSP-513 at 0.03 mg/kg, ■) and 8.3 ± 0.8 ml/min (NSP-513 at 0.1 mg/kg, ▲). Data represent the mean ± S.E.M. of 5 animals per group. **P<0.01 and *P<0.05 versus vehicle. The sham-operation group received vehicle without the application of current.

DISCUSSION

We found that NSP-513 was a highly potent and selective PDE 3 inhibitor. In particular, NSP-513 was 850 times more potent against PDE 3 than against the other PDE isozymes (1, 2, 4 and 5), and the selectivity of NSP-513 for PDE 3 over the other PDE isozymes was much greater than that of cilostazol (11 times) or dipyridamole (non-selective for PDE 3). Since PDEs exist in multiple forms in cells and tissues and each form of PDE acts in a complex manner in physiological responses, the extremely high PDE 3 selectivity of NSP-513 suggests that this compound would be useful for assessing the physiological role of PDE 3.

In our in vitro platelet aggregation study, NSP-513 dis-

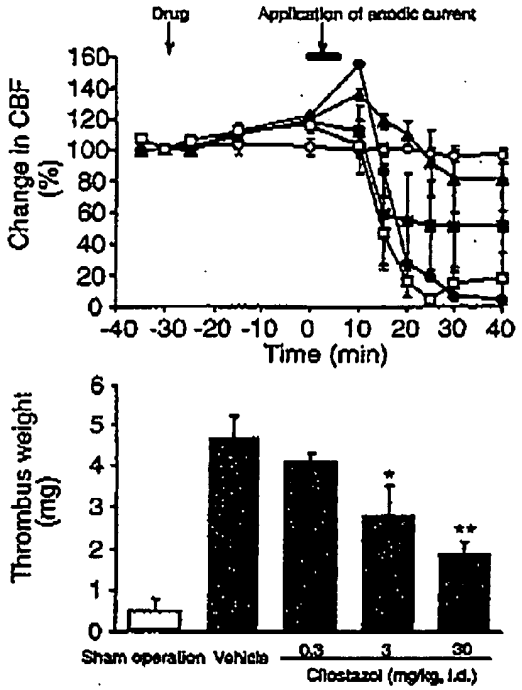

Fig. 2. Effects of intraduodenally (i.d.) injected cilostazol on carotid arterial blood flow (CBF) (top) and thrombus weight (bottom) in an electrical stimulation-induced carotid arterial thrombosis rat model. Cilostazol (0.3–30 mg/kg, i.d.) or vehicle was injected 30 min before the application of anodic current (1 mA, 6 min). Changes in CBF are expressed as a percentage of the basal CBF measured just before injection in each rat. The basal CBF values were 7.2 ± 1.1 ml/min (sham ope., ○), 6.5 ± 0.8 ml/min (vehicle, □), 5.3 ± 0.1 ml/min (cilostazol at 0.3 mg/kg, ●), 5.2 ± 0.4 ml/min (cilostazol at 3 mg/kg, ■) and 8.2 ± 0.7 ml/min (cilostazol at 30 mg/kg, ▲). Data represent the mean ± S.E.M. of 4 or 5 animals per group. **P<0.01 and *P<0.05 versus vehicle. The sham-operation group received vehicle without the application of current.

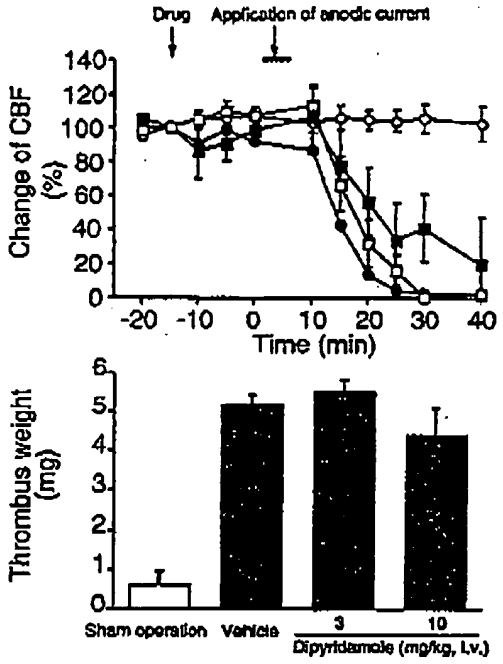

Fig. 3. Effects of i.v. injected dipyridamole on carotid arterial blood flow (CBF) (top) and thrombus weight (bottom) in an electrical stimulation-induced carotid arterial thrombosis rat model. Dipyridamole (3 and 100 mg/kg, i.v.) or vehicle was injected 15 min before the application of anodic current (1 mA, 6 min). Changes in CBF are expressed as a percentage of the basal CBF measured just before injection in each rat. The basal CBF values were 6.4 ± 1.0 ml/min (sham ope., ○), 6.8 ± 0.8 ml/min (vehicle, □), 7.0 ± 1.0 ml/min (dipyridamole at 3 mg/kg, ●) and 6.5 ± 1.1 ml/min (dipyridamole at 10 mg/kg, ■). Data represent the mean ± S.E.M. of 5 animals per group. The sham-operation group received vehicle without the application of current.

played potent and concentration-dependent inhibition of platelet aggregation induced by several platelet activators; furthermore, NSP-513 exhibited little or no species difference in its antiplatelet activity, at least with respect to human versus rat platelet aggregation induced by collagen. However, Kimura et al. (14) reported that the inhibitory potencies of cilostazol on the ADP-induced platelet aggregation in rats is relatively less than that in humans. Therefore, it is considered that NSP-513 exhibits different potencies against human and rats platelet aggregation induced by other platelet activators such as ADP, and NSP-513 should be used with caution. In the pulmonary thromboembolism model, orally administered NSP-513 attenuated the rate of mortality induced by several types of platelet activators such as arachidonic acid, ADP, collagen and epinephrine. In contrast, aspirin even at doses of 300 or 900 mg/kg caused only partial improvement in the rate of mortality induced by ADP or by collagen plus epinephrine. It has been reported that the lethal effects of platelet activators such as arachidonic acid, ADP, collagen and epinephrine in the pulmonary thromboembolism model are caused by massive occlusion of the microcirculation of the lung by platelet thromboemboli (23–25). Therefore, our results suggest that a selective PDE 3 inhibitor displays a broad spectrum of antithrombotic activities in in vivo thrombus formation as well as in in vitro platelet aggregation, and that the antiplatelet and antithrombotic actions of a selective PDE 3 inhibitor are distinctly different from those of a COX inhibitor such as aspirin.

Electrical stimulation is frequently used to induce occlusive arterial thrombosis in rats (17–22, 26). Several reports

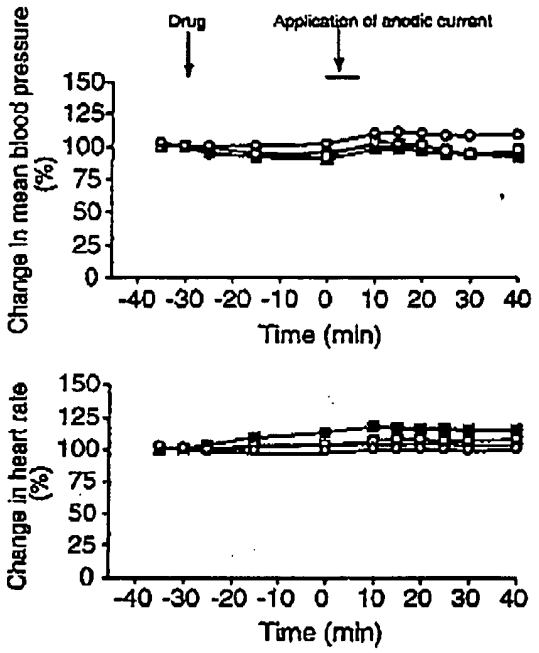

Fig. 4. Effects of intraduodenally injected NSP-513 on mean blood pressure (top) and heart rate (bottom) in an electrical stimulation-induced carotid arterial thrombosis rat model. NSP-513 (0.01–0.1 mg/kg, i.d.) or vehicle was injected 30 min before the application of anodic current (1 mA, 6 min). Changes in mean blood pressure and heart rate are expressed as a percentage of the basal values measured just before injection in each rat. The basal mean blood pressure values were 113 ± 4 mmHg (vehicle, ○), 114 ± 1 mmHg (NSP-513 at 0.01 mg/kg, ●), 118 ± 3 mmHg (NSP-513 at 0.03 mg/kg, □) and 119 ± 6 mmHg (NSP-513 at 0.1 mg/kg, ■). The basal heart rate values were 344 ± 20 beats/min (vehicle), 341 ± 11 beats/min (NSP-513 at 0.01 mg/kg), 350 ± 8 beats/min (NSP-513 at 0.03 mg/kg) and 357 ± 29 beats/min (NSP-513 at 0.1 mg/kg). Data represent the mean ± S.E.M. of 5 animals per group.

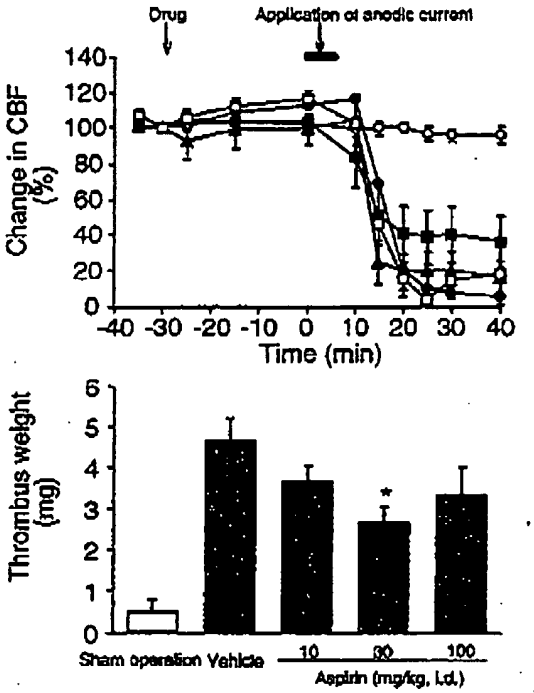

Fig. 5. Effects of intraduodenally (i.d.) injected aspirin on carotid arterial blood flow (CBF) (top) and thrombus weight (bottom) in an electrical stimulation-induced carotid arterial thrombosis rat model. Aspirin (10–100 mg/kg, i.d.) or vehicle was injected 30 min before the application of anodic current (1 mA, 6 min). Changes in CBF are expressed as a percentage of the basal CBF measured just before injection in each rat. The basal CBF values were 7.2 ± 1.1 ml/min (sham ope., ○), 6.5 ± 0.8 ml/min (vehicle, □), 8.6 ± 1.0 ml/min (aspirin at 10 mg/kg, ●), 7.8 ± 1.2 ml/min (aspirin at 30 mg/kg, ■) and 7.5 ± 1.7 ml/min (aspirin at 100 mg/kg, ▲). Data represent the mean ± S.E.M. of 5 animals per group. *P<0.05 versus vehicle. The sham-operation group received vehicle without the application of current.

have suggested that electrical stimulation-induced injury of blood vessels produces experimental thrombus with a morphology that is consistent with human pathology (17, 20, 26). In our rat model of electrical stimulation-induced carotid arterial thrombosis, i.d. injection of aspirin produced significant antithrombotic effects that were not dose-related. Aspirin is the most commonly used antiplatelet drug and has been shown to inhibit not only the production of proaggregatory TXA_2 in platelets but also the production of antiaggregatory prostaglandin (PGI_2) in vessel walls; this phenomenon is known as the "aspirin dilemma" (27–32). Therefore, our results suggest that the ability to synthesize PGI_2 is not completely abolished in the carotid arterial wall following electrical stimulation and that the dose-independent antithrombotic effects of aspirin may be due to the "aspirin dilemma." Heparin, an anticoagulant drug, also inhibited thrombus formation when given at a dose of 300 units/kg per hour, which maximally increased the activated partial thromboplastin time (APTT) from 19 ± 2 to >300 s (H. Hirose et al., unpublished data). Schumacher et al. (21, 26) demonstrated that heparin (300 units/kg, i.v., every 30 min) significantly prevented carotid arterial thrombus formation in rats in response to electrical stimulation and that the % inhibition with heparin was 50–63%. These previous data for heparin seem to be consistent with our data. Thus, this rat thrombosis model appears to be appropriate for the evaluation of both antiplatelet and anticoagulant drugs. In this rat model, we found that NSP-513 dose-dependently reduced thrombus formation on the inner surface of the ar-

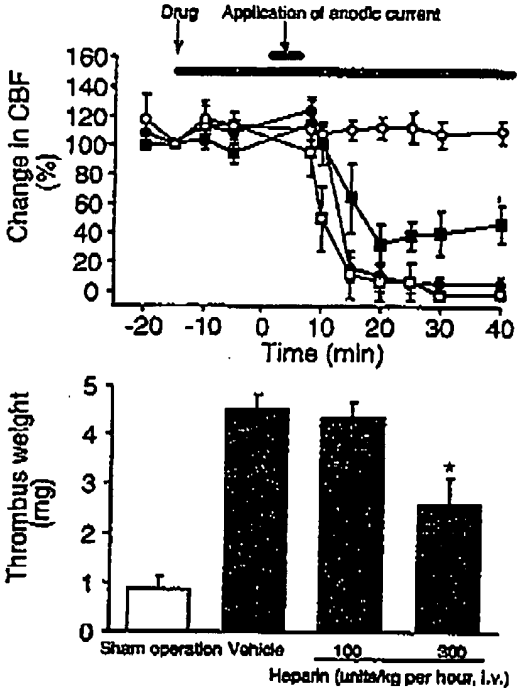

Fig. 6. Effects of i.v. infusion of heparin on carotid arterial blood flow (CBF) (top) and thrombus weight (bottom) in an electrical stimulation-induced carotid arterial thrombosis rat model. Infusion of heparin (100 and 300 units/kg per hour) or vehicle was started 15 min before the application of anodic current (1 mA, 6 min). Changes in CBF are expressed as a percentage of the basal CBF measured just before infusion in each rat. The basal CBF values were 7.6 ± 1.7 ml/min (sham ope., ○), 5.7 ± 0.8 ml/min (vehicle, □), 5.6 ± 0.7 ml/min (heparin at 100 units/kg per hour, ●) and 5.3 ± 1.1 ml/min (heparin at 300 units/kg per hour, ■). Data represent the mean ± S.E.M. of 4 or 5 animals per group. *P<0.05 versus vehicle. The sham-operation group received vehicle without the application of current.

terial vessel wall, although antithrombotic effects of selective PDE 3 inhibition were not reported previously using the electrical stimulation-induced injury model. At a dose of 0.1 mg/kg (i.d.), 75% inhibition of thrombus formation (weight) was observed. As a result, effective doses of NSP-513 were lower in our rat model than in our mouse model. Antiplatelet potency of NSP-513 is affected by the type of platelet-aggregating agents used (Table 2). Also, platelet aggregation in the mouse model is induced by systemic application (i.v.) of the aggregating agent; and, in contrast, in the rat model, it is induced by local stimulation into the carotid artery. Therefore, one possible explanation for the difference in effective doses between the rat and mouse models involves the different experimental conditions with respect to the type and mode of application of aggregating agents and/or stimulation. Of course, we cannot rule out the possibility of a species difference between the rat and mouse in the antiplatelet potency and/or pharmacokinetics of NSP-513. Furthermore, the antithrombotic activity of NSP-513 was at least 300 times more potent than that of cilostazol in two in vivo thrombosis models, although the antiplatelet activity of NSP-513 was 11 to 65 times more potent than that of cilostazol in in vitro platelet aggregation assays. We confirmed the oral absorbability of NSP-513 in our preliminary pharmacokinetic study using rats, and the maximum plasma concentration of NSP-513 was 750 ng/ml at 0.5 h after a dose of 3 mg/kg (p.o.). In contrast, it has been reported that the maximum plasma concentration of cilostazol is about 750 ng/ml at 2–4 h after the administration of 10 mg/kg (p.o.) (33). Therefore, one reason for the difference in potency between the in vitro and in vivo conditions may be the lower oral absorbability of cilostazol compared with that of NSP-513. In addition, it has been reported that the concentration of cilostazol after oral administration differs among several cardiovascular sites including the blood, aorta, vena cava and heart (33). Further antithrombotic studies including assay of the pharmacokinetics of NSP-513 would be necessary to resolve this issue.

On the other hand, we did not observe any significant antithrombotic effects of dipyridamole at intravenous doses of 3 and 10 mg/kg. Since 0.01 mg/kg (i.d.) of NSP-513 produced a significant reduction in thrombus weight, the in vivo antithrombotic activity of dipyridamole appears to be roughly 1,000 times less than that of NSP-513. Dipyridamole was at least 100 times more selective against human platelet PDE 2 and 5 than against human platelet PDE 3. The inhibitory effect of dipyridamole on human platelet PDE 3 was 2,600 times less than that of NSP-513. Other investigators have reported that the IC_{50} values (μM) of dipyridamole for human PRP aggregation induced by ADP, collagen and arachidonic acid were 210, 380 and >1000, respectively (34), and that dipyridamole (5 mg/kg, i.v.) did not prevent arterial thrombus formation in an electrical stimulation-induced injury model in rats (21). Therefore, our findings strongly support the concept that the activity of PDE 3 is the primary contributor to human platelet aggregation, despite the presence of other PDEs such as PDE 2 and 5.

In addition, we did not observe any significant changes in heart rate or blood pressure even at antithrombotic doses of NSP-513 (0.01–0.1 mg/kg, i.d.). PDE inhibitors elicit cardiovascular effects, including relaxation of vascular smooth muscle and positive inotropic responses in cardiac muscle (35–37). Interestingly, in rat cardiac muscle, it has been reported that selective inhibitors of PDE 3 and PDE 4 do not increase the force of contraction when given alone, but such agents do increase contractility when

administered in combination (38). Therefore, one reason for the lower cardiovascular activity of NSP-513 may be that the inhibition of total PDE activity is more PDE 3-sensitive in platelets than in cardiac muscle or vascular smooth muscle in rats. However, since it has been predicted that the composition of the cardiac PDE isozymes in rats differs from that in other species including humans (10), further cardiovascular studies on NSP-513 are necessary.

In conclusion, the inhibition of PDE 3 alone by NSP-513 has the potential to prevent not only in vitro platelet aggregation but also in vivo thrombosis formation induced by various platelet-aggregating agents or electrical stimulation in the arterial vessel wall, and the highly selective PDE 3 inhibitory effect of NSP-513 suggests that this compound would be useful for assessing the physiological role of PDE 3. In addition, since the antiplatelet and antithrombotic activities of NSP-513 are greater than those of cilostazol, dipyridamole or aspirin, NSP-513 may have value in the treatment of arterial thrombotic disorders.

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